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COPPER TOXICITY IN *BUSYCON CANALICULATUM* L.

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Copper is among the most toxic of the heavy metals to most organisms (Bowen, 1966; Bryan, 1971), and it may be introduced in significant amounts into the coastal marine environment from industrial sources. It thus seemed desirable to examine the tolerance for copper of a commercial organism commonly found in estuarine and coastal regions, the channeled whelk, *Busycon canaliculatum*. The effects of high copper concentrations on marine prosobranch gastropods such as *Busycon* are of particular interest, because these snails (like several other groups of molluscs and arthropods) normally accumulate and store copper and use it in the synthesis of the blood pigment, hemocyanin. Studies of the copper metabolism of *Busycon* under normal environmental conditions have been described elsewhere (Betzer, 1972; Betzer and Pilson, 1974). This paper presents a series of experiments carried out to determine the toxic concentration of copper for *Busycon* and to investigate the effects of high copper concentrations by determination of tissue copper concentrations, by tracing uptake with ^{64}Cu, and by histological examination.

Materials and Methods

Specimens of *Busycon canaliculatum* collected in pots from the Wickford-Fox Island region of Narragansett Bay, Rhode Island, were placed, 2 or 3 whelks per tank, in 8-liter all-glass aquaria. The tanks were filled with bay water to which various volumes of a cupric chloride stock solution had been added, so that the final concentration of added copper was between 0 and 1000 μg/l. The tanks were covered, aerated, and incubated in a wet table of running bay water to maintain the same temperature as in the natural environment (from 13–15° C in early June to 20–22° C in August). Every three to four days the water was changed and fresh copper stock was added. Animals that died were removed for copper determination. The whelks were not fed, unless otherwise noted.

For tissue copper determinations, exposed and control whelks were bled and dissected. Blood and tissue samples (gut, digestive gland, kidney, gonad, gills, osphradium, and foot muscle) were digested with aliquots of a solution prepared by mixing 100 ml of concentrated perchloric acid and 400 ml of concentrated nitric acid. The samples were analyzed for copper by the spectrophotometric cuproine method (b) of Diehl and Smith (1958) or by atomic absorption spectroscopy, as described elsewhere (Betzer and Pilson, 1974).

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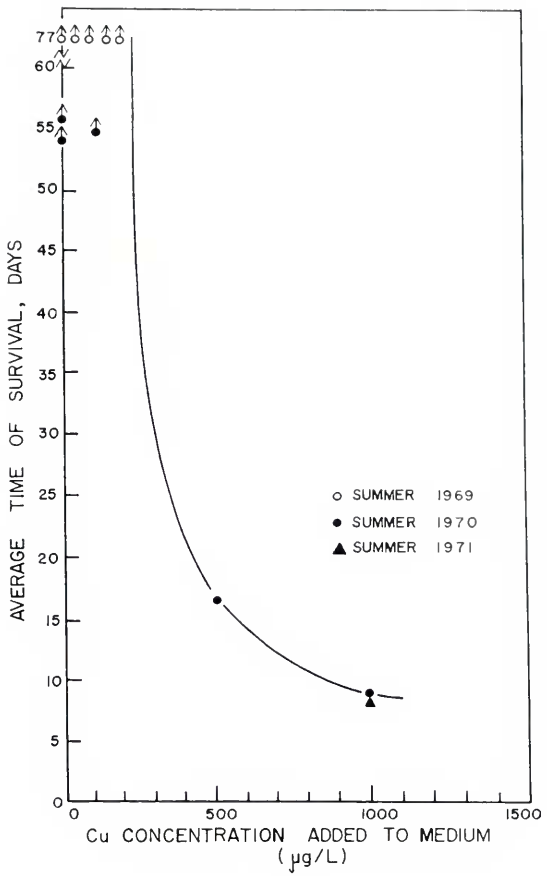

FIGURE 1. Average time of survival for whelks exposed to various copper concentrations; open circles, average time of survival for 6 whelks exposed to each concentration, summer 1969; closed circles, average time of survival for 4 whelks exposed to each concentration, summer 1970; closed triangles, average time of survival for 8 whelks exposed to 1000 µg/l added copper, summer 1971. Arrow indicates that the whelks were still alive when the experiment was terminated.

The sequence of uptake of copper into the tissues from both toxic (470 µg/l) and nontoxic (6 µg/l) solutions was followed using radioactively labeled ^{64}Cu added to bay water, from which trace metals had previously been removed by passage through a Chelex column (Riley and Taylor, 1968). Individual whelks were held in polypropylene beakers containing 3 liters of labeled solution for 6, 24, and 48 hr. After incubation the whelks were counted whole above a sodium-iodide crystal for gamma emission due to copper, using a multichannel analyzer. The whelks were dissected, and blood and tissue samples were also counted to determine ^{64}Cu uptake as described previously (Betzer, 1972).

Tissues of control whelks and whelks exposed to toxic copper concentrations (1000 µg/l added copper) for various periods of time were examined for evidence

TABLE 1.

Copper concentrations (μg/g fresh weight) of gills and osphradia of whelks exposed to various Cu concentrations

	# of whelks	Added Cu μg/l	Days exposure	Gills		Osphradium	
				$\bar{X}$ ± S.D.	range	$\bar{X}$ ± S.D.	range
July–August 1970	4	0	54	35± 6	29– 41	16± 0.7	15– 16
	4	100	54	43± 10	32– 54	25± 4	23– 30
	4	500	$\bar{X}$ =16 (all died)	316±158	215–551	96±40	64–151
	4	1000	$\bar{X}$ = 9 (all died)	231± 73	128–298	78±31	38–108
August 1971	3	0	0	77± 11	66– 89	45±10	39–56
	4	0	9	89± 22	58–107	40±10	27–52
	3	1000	4*	112± 16	96–128	60±22	35– 73
	4	1000	7 (one died)	188± 90	55–246	112±18	94–134
	4	1000	9 (three died, one dying)	185± 40	129–216	116±18	92–131

* transferred to an aquarium without added copper for a few hours before dissection.

of histopathology. Tissues were normally fixed in a modified Zenker-formol fixative, routinely processed, cut to a thickness of 6 μ, and stained with hematoxylin and eosin. For a histochemical study of copper deposition, the tissues were prepared according to the rubeanic acid method of Uzman (1956).

RESULTS

Copper tolerance of Busycon

In a preliminary experiment in June, 1969, a total of 30 whelks were incubated in 10 tanks containing 0, 50, 100, 150, and 200 μg/l added copper, with 2 tanks at each concentration. These concentrations were chosen because Marks (1938) had found that in similar experiments the limits of Cu tolerance for all gastropods tested were 100–200 μg/l. Four times during the 77-day course of the experiment, blood samples were removed from each animal in connection with another study. A small quahog was added to each tank as food on days 62, 72, and 74. During the course of the study, the mortality in the high copper tanks (150 and 200 μg Cu/l) was 50%, the same as in the control tanks (no added copper). Thus it seemed that the high ionic copper concentration of the medium was probably not responsible for the whelk deaths, but that death was more likely due to infection or injury during bleeding.

In a second experiment begun in July, 1970, 16 whelks were incubated, 2 per tank, in a broader range of added copper concentrations: 0, 100, 500, and 1000 μg/l, with 2 tanks at each concentration. After 54 days the experiment was terminated, and all the whelks were dissected for tissue copper determinations. All whelks at 0 and 100 μg/l survived the entire experiment and could often be seen actively crawling about the tank, adhering to the glass walls. During exposure to the higher copper concentrations, however, whelks frequently remained withdrawn into their shells, emitting large quantities of mucus. Figure 1 presents

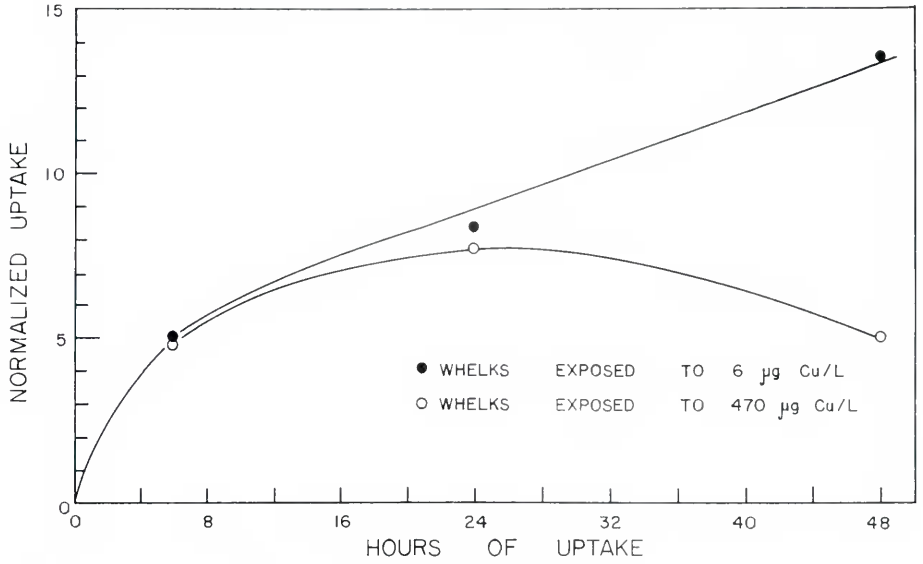

FIGURE 2. Uptake of ^{61}Cu by whole whelks, expressed as:

$$\text{Normalized uptake} = \frac{\mu\text{g Cu taken up per g of whelk}}{\mu\text{g/ml Cu initially present in medium}};$$

closed circles, whelks exposed to 6 µg/l added copper; open circles, whelks exposed to 470 µg/l added copper.

the average time of survival for whelks at various copper concentrations in this experiment, as well as in the preliminary experiment of the previous summer, and for whelks exposed to 1000 µg/l copper in August, 1971. In 1970, at 500 µg/l, the whelks survived an average of 16.5 days (range = 11–27 days); at 1000 µg/l, an average of 9 days (range = 7–10.5 days). In 1971, whelks exposed to 1000 µg/l added copper, and removed at intervals for chemical and histological copper determinations, survived 8 days (range = 7–9 days). Thus for these periods of exposure (54 and 77 days) the tolerance limit of *Busycon* for copper is between 200 and 500 µg/l.

Tissue copper concentrations of whelks exposed to various copper concentrations

Copper concentrations were determined for the 16 whelks exposed to various concentrations in the 1970 toxicity study and for 18 whelks exposed to 0 and 1000 µg/l added copper in August, 1971. In the 1971 experiment, 11 whelks exposed to 1000 µg Cu/l were dissected after incubation periods of 4 days (3 whelks), 7 days (4 whelks), and 9 days (4 whelks); control whelks were dissected after incubation periods of 0 days (3 whelks) and 9 days (4 whelks). In both experiments, whelks exposed to toxic copper concentrations showed marked increases in the amount of copper on the osphradium and, particularly, the gills (Table I). In 1970 there was no significant increase in whelks exposed to a high but sublethal copper concentration (100 µg/l), but there was a striking increase in the whelks

TABLE II

Concentration of uptaken ^{64}Cu and normalized uptake in tissues of whelks exposed to 6 and 470 μg Cu/l

Tissue	Medium	μg Cu taken up/g fresh weight			Normalized uptake*		
		6 hr	24 hr	48 hr	6 hr	24 hr	48 hr
Gills	low Cu:	0.29	0.49	0.18	48	82	30
	high Cu:	31	30	37	66	64	79
Osphradium	low Cu:	0.40	1.1	0.36	67	187	61
	high Cu:	37	27	32	79	58	68
Kidney	low Cu:	0.056	0.13	0.11	9.3	22	18
	high Cu:	1.3	2.0	2.7	2.8	4.4	5.9
Gut	low Cu:	0.041	0.22	0.37	6.8	37	61
	high Cu:	0.88	1.3	2.2	1.9	2.8	4.8
Digestive Gland	low Cu:	0.04	0.14	0.30	6.7	24	49
	high Cu:	0.39	0.43	0.72	0.84	0.93	1.5
Blood	low Cu:	0.017	0.029	0.037	2.9	4.9	6.1
	high Cu:	0.61	0.33	0.35	1.3	0.71	0.75

* Normalized uptake = $\frac{\text{μg Cu taken up per gram whelk}}{\text{initial Cu concentration of medium, μg/ml}}$

that died from copper toxicity at 500 and 1000 μg/l. In 1971, concentrations were increased after 4 days of exposure to 1000 μg Cu/l and more than doubled after 7 and 9 days. The differences in concentration between the control groups of the 2 experiments were probably due to differences in time of capture (July of 1970 and August of 1971) and feeding histories (Betzer and Pilson, 1974).

Exposure of whelks to high copper concentrations had no apparent effect on the copper concentrations in the blood, kidney, or muscle, which showed quite similar concentrations in all groups within an experiment. In the case of the gut, gonad, and particularly the digestive gland, which has a copper-storing function, there was tremendous individual variation in the copper content within experimental groups—as much as 10-fold. This variation is characteristic of *Busycon* (Betzer and Pilson, 1974) but it obscured possible copper accumulation resulting from exposure to high copper concentrations.

^{64}Cu uptake by Busycon *from a toxic concentration*

Previous work has shown that *Busycon* can take up labeled copper from nontoxic seawater solutions containing 6–100 μg/l (Betzer, 1972). In the present experiment, 3 whelks were also exposed to toxic concentrations (470 μg/l) which caused them to withdraw into the shell and emit mucus even within the short periods of incubation used (6, 24, and 48 hr). Figure 2 presents uptake by the whole whelks, expressed as the ratio of μg of copper taken up per gram of whelk to the initial copper concentration of the incubation medium ("normalized up-

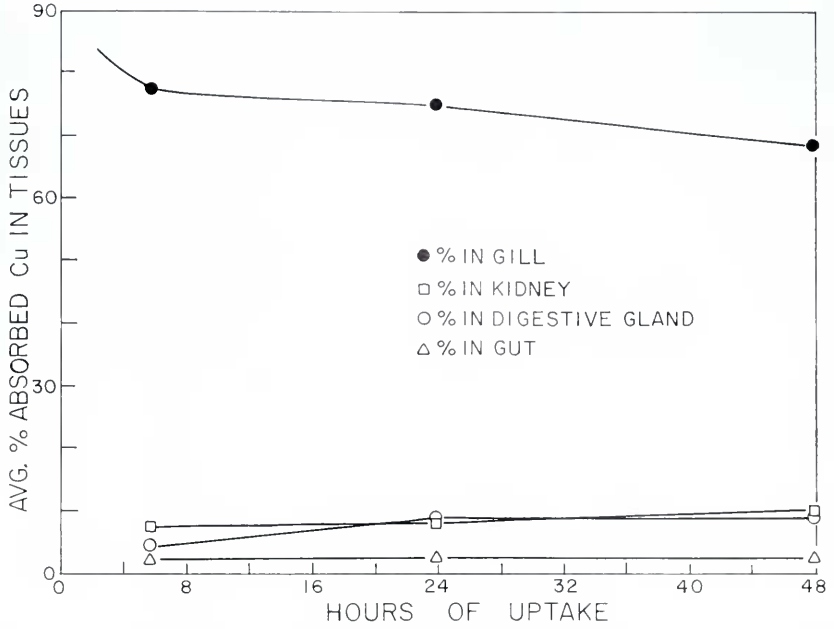

FIGURE 3. Sequence of copper uptake into gill and most important visceral tissues with time from a concentration of 470 μg/l. The total amount of copper taken up into the gill, osphradium, digestive gland, gut, kidney, and gonad was summed and the percentage found in each tissue was plotted for each uptake period; closed circles, percentage in gill; squares, percentage in kidney; open circles, percentage in digestive gland; open triangles, percentage in gut.

take"); results of uptake by 3 whelks exposed to low, environmental copper concentrations are plotted for comparison. Until 24 hr, whelks exposed to both copper concentrations showed increasing concentrations of labeled copper, but there was a decrease by 48 hr in the whelk exposed to toxic copper. In the low-copper whelks in this experiment, the shell accounted for an average of 44% of the total copper taken up; but the shell of the whelks at the toxic concentration accounted for an average of 78% of the copper taken up. Thus, at the toxic concentration, a smaller proportion of the uptaken copper was in or on the soft tissues.

Copper uptake by individual soft tissues is presented in Table II. Uptake of copper by the gills and osphradia of whelks in the medium with toxic copper was on the order of 50–80 times the initial concentration of the medium, similar to that of whelks exposed to low concentrations of added copper. In the whelks exposed to low copper concentrations, uptake by the gill and osphradium was high at 6 hr, increased until 24 hr, and then decreased again by 48 hr, as ^{64}Cu in the medium was depleted. The normalized uptake of gills and osphradia at the toxic concentrations remained about the same from 6 hr through 48 hr.

The internal tissues and organs, on the other hand, generally increased in concentration of absorbed copper in both groups. The quantities of labeled copper taken up were larger in the whelks exposed to toxic concentrations; but in both

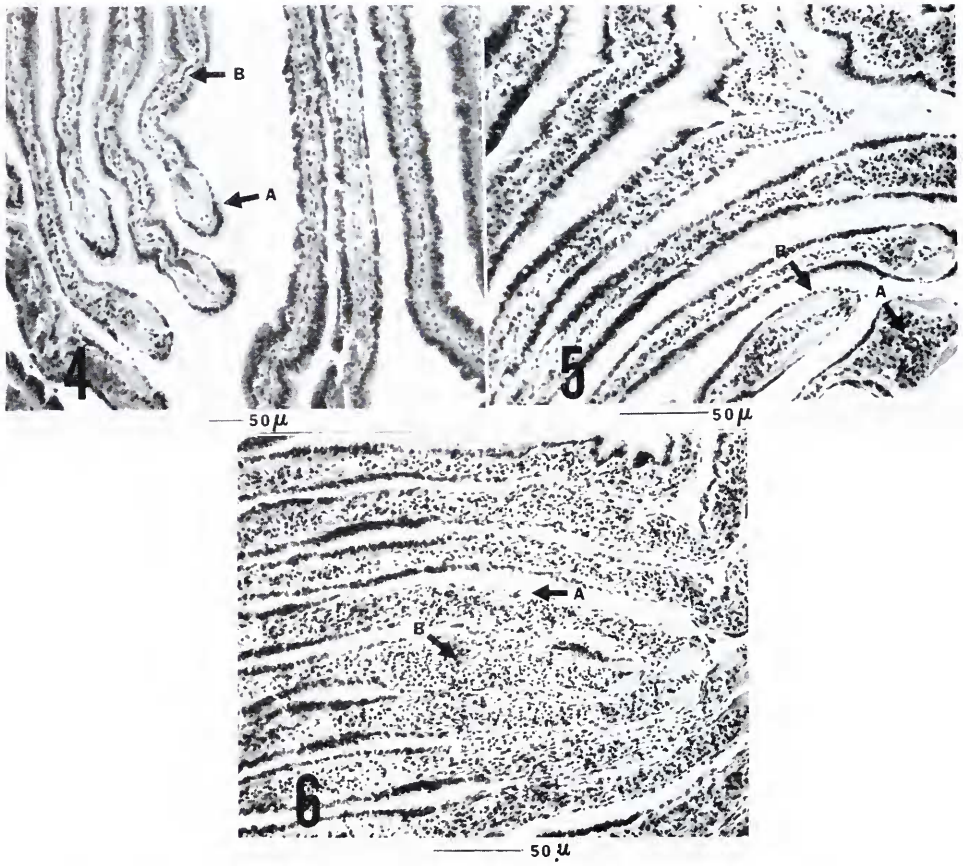

FIGURE 4. Gills of a control whelk, showing leaflet (Arrow A) structure of the organ. The inner area (Arrow B) of the leaflet shows normal blood lacunae; Zenker's, H & E.

FIGURE 5. Gills of a whelk exposed 3 days to toxic copper, showing dilation of blood sinuses filled with blood cells (Arrow A). The mucosa at the tips of the leaflets shows some regions of necrosis and sloughing (Arrow B); Zenker's, H & E.

FIGURE 6. Gills of a whelk exposed 7 days to toxic copper, showing extensive necrosis of the swollen gill leaflets (Arrow A), which lack epithelium, and amebocytic infiltration of the whole area (Arrow B); Zenker's, H & E.

groups, the amount taken up was small in comparison to the amount present in the tissues. The uptake normalized for initial concentration of the medium was 2–33 times lower, however, in whelks exposed to 470 μg/l than in those exposed to 6 μg/l. After 48 hr, the kidney, gut, digestive gland, and blood of whelks in the toxic medium had not yet reached the normalized uptake shown at 6 hr by whelks taking up copper from low concentrations. Thus the rate of uptake of labeled copper into these internal tissues from the toxic medium was not proportional to the rate of accumulation on the gills.

To follow the sequence of copper uptake into important tissues of the mantle cavity and visceral mass, the total labeled copper taken up into the gills, osphradium,

digestive gland, gut, kidney, and gonad can be summed, and the percentage found in the various tissues computed for each uptake period. (The blood is omitted because the blood volume was not known.) In whelks exposed to 6 μg/l labeled copper, the gill had 90% of the uptaken copper after 1 hr; but as copper entered the body, the gill decreased in importance so that by 48 hr it contained only 15% of the total. Meanwhile, the digestive gland showed a steady increase in accumulation so that at 48 hr it contained 50% of the uptaken copper. This is the typical pattern for *Busycon* at normal environmental copper concentrations (Betzer and Pilson, 1975). Figure 3 is a graph of uptake into gills and tissues of the visceral mass for the 3 whelks incubated in 470 μg/l added copper. Here, the gill also had a high percentage of the total uptaken copper early in the incubation, but it maintained its importance throughout the 48-hr period. The digestive gland did not show the dramatic rise in copper accumulation that occurs in normal copper concentrations. Thus these data also suggest that proportionately less copper is being taken into the body from the gill.

Histopathologic findings for whelks exposed to toxic copper

The tissues of 3 control whelks and whelks exposed to 1000 μg/l added copper for 3 days (4 whelks), 4 days (2 whelks), 5 days (4 whelks), and 7 days (3 whelks) were examined for evidence of histopathology. Animals examined after 3 days of exposure to toxic levels of copper (Figure 5) showed dilated efferent blood sinuses and blood lacunae in the leaflets of the gill in comparison with control whelks (Figure 4). The blood sinuses were also dilated in the leaflets of the osphradium. The dilated areas were filled with a pink-staining material and showed a tremendous increase in amebocytes. This may be considered a type of inflammatory response in *Busycon*. There were also noted, in a few leaflets of the gills, focal areas of necrosis and sloughing of epithelial cells.

With increased exposure time (4, 5, and 7 days) there was a progressive increase in the swelling of the leaflets and in the inflammatory response, and in the necrosis and sloughing of the epithelium of the osphradium and gills (Fig. 6). The leaflets were in some cases completely denuded of epithelium, leading to necrosis of the various structures of the septum. At times the tips of the denuded septum ballooned out.

Microscopic examination of the heart, kidney, digestive gland, salivary glands, gut, foot, radula, reproductive tract, and mantle did not show any changes which could be attributed to the action of copper. Histochemical study of all tissues for copper deposition according to the method of Uzman (1956) did not show any differences between exposed and control animals. This does not seem to be a useful technique for studying copper deposition in *Busycon*.

Discussion

The limits of copper tolerance for *Busycon*, between 200 and 500 μg/l, are high in comparison to the concentrations encountered in the natural environment; unpolluted Narragansett Bay water has a concentration of 3 μg/l (D. Hallett, University of Rhode Island, Graduate School of Oceanography, personal communication). Other mollusks have shown a greater sensitivity to copper than *Busycon*.

Marks (1938) found in experiments similar to those described here that for 10 species of Pacific coast mollusks the upper limit of copper tolerance was 100–200 μg Cu/l; except for one species of clam, none survived more than 18 days at 200 μg/l added copper. This was the same toxicity threshold found for *Mytilus edulis* (Scott and Major, 1972). Shuster and Pringle (1968) exposed quahogs (*Mercenaria mercenaria*) to copper concentrations of 25 and 50 μg/l and found 63% and 78% mortality by the 15th week, although oysters showed only 10% and 15% mortality after 20 weeks at the same concentration. Harry and Aldrich (1963) found that the freshwater pulmonate snail, *Taphius glabratus*, showed distress after only 24 hr of exposure to concentrations of 50–100 μg/l added copper.

As discussed by Bryan (1971), temporary storage of a metal in a particular tissue is a method of removing it from the rest of the body, and consequently reducing its toxic effects. Such accumulation of ^{64}Cu by the digestive gland is seen in *Busycon* exposed to labeled solutions of low (6 μg/l), non-toxic concentrations; and this, followed by later excretion of copper, may be the mechanism by which whelks resist higher copper concentrations.

Yager and Harry (1964) exposed *Taphius glabratus* to concentrations of labeled copper which allowed normal behavior and to concentrations which caused distress; they found that the livers of distressed snails contained less absorbed labeled copper than those of normal snails, and concluded that distress was somehow produced by disruption of membrane permeability, although the site of copper absorption was not identified. In *Busycon*, experiments using ^{64}Cu show that under normal concentrations, copper is taken up and transferred to the internal tissues; at toxic copper concentrations, where only the gills (and osphradium) show tissue damage, the rate of transfer into the internal tissues, particularly the digestive gland, is sharply decreased. These results indicate that the gills are the primary site of entrance of dissolved Cu into the whelk body, rather than other possible routes such as through the gut or the general body surface.

The histopathologic findings in *Busycon* are much the same as those seen in previous studies by Yevich (unpublished) after the exposure of the fish, *Fundulus heteroclitus*, and the quahog, *Mercenaria mercenaria*, to toxic concentrations of copper. There, too, the main organ involved was the gill, in which there was necrosis and sloughing of the epithelium, and, in *Fundulus*, a ballooning of the tips of the gill filaments. In *Busycon*, the clear evidence of structural damage to the gill at toxic concentrations suggests that there may be interference with respiration, as well as with normal copper transport mechanisms into the body.

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Summary

1. The effects of high concentrations of copper in seawater upon *Busycon canaliculatum* were followed histologically, by determination of tissue Cu concentrations, and by tracing uptake with radioactively labeled copper (^{64}Cu).

2. Whelks showed a high resistance to ionic Cu, with a tolerance limit between 200–500 μg/l at normal habitat temperatures for the exposure periods used (54–77 days).

3. At lethal concentrations, Cu was accumulated at the gill and osphradium; and these tissues also showed progressive histopathologic change, consisting of swelling of the gill filaments, amebocytic infiltration of the connective tissue, and necrosis and sloughing of the mucosa.

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